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Introns Are Inconsequential to Efficient Formation of Cellular Thymidine Kinase mRNA in Mouse L Cells

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TK **mRNA levels were determined in mouse L cells transformed with intron deletion mutations of the chicken *TK* gene. Whether normalized per cell, per integrated gene, or per internal control signal, intron deletion did not diminish the efficiency of *TK* mRNA formation in transformed L cells. The results demonstrated that introns are not required for efficient biogenesis of cellular mRNA in transformed mouse L cells.**

The general importance of introns for efficient gene expression in mammalian cells is an unresolved issue. Early work with recombinant simian virus 40 showed convincingly that efficient formation of viral 16S mRNA requires the presence of an intron in the DNA template (10, 12, 13, 15, 16); the intron requirement was manifested at a posttranscriptional level and could be satisfied by substituting an intron from a heterologous gene. These results suggested that passage through a splicing pathway might be a general requirement for formation of stable cytoplasmic mRNA. Such a requirement could explain the poor transformation efficiency of various intronless minigenes (5, 16, 17). However, rigorous reaffirmation of the importance of introns to eucaryotic mRNA formation has not been reported. In fact, for certain viral, plant, and yeast genes, evidence to the contrary has accumulated. Wild-type and intronless derivatives of the genes encoding adenovirus E1A protein (2, 25), polyomavirus T antigens (26, 27), and the Rous sarcoma virus envelope protein (3) were equally efficient in generating mRNA in infected cells. Similar results were obtained for bean phaseolin in transformed plants (4) and *Saccharomyces cerevisiae* actin in transformed *S. cerevisiae* (21). Given these exceptions, a careful investigation of the importance of introns to expression of cellular genes in mammalian cells was warranted.

Direct comparison of mRNA levels in mammalian cells transformed with wild-type and intronless cellular genes has not been reported. Hofbauer et al. (14) achieved expression of an intronless mouse thymidine kinase (*TK*) cDNA by using a herpesvirus *TK* promoter but did not compare expression levels with that of an intact gene. Evidence suggestive of an intron requirement for expression of mouse dihydrofolate reductase (*DHFR*) was reported by Lee et al. (16) and Gasser et al. (5), who showed that intron-free *DHFR* minigenes were less efficient than intron-containing minigenes in transforming DHFR$^-$ rodent cells to methotrexate resistance. Similarly, Lewis (17) noted that an intron-free hamster *TK* minigene was 10-fold less efficient than intron-containing minigenes in transforming TK$^-$ mouse L cells to hypoxanthine-aminopterin-thymidine (HAT) resistance. However, transformation efficiency is an indirect and potentially inaccurate measure of gene function. Furthermore, because of the large size of the mammalian *DHFR* and *TK* genes (due to the presence of large introns), direct comparison of intronless gene function with wild-type gene function was not feasible.

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To investigate whether introns were required for efficient expression of cellular genes in animal cells, a series of intron deletion mutations of the chicken *TK* gene were constructed and transformed into L cells, and their level of expression was quantitated. The full-length chicken *TK* gene is interrupted by six introns. A seventh intron, in the 3′ nontranslated region, is removed from rare *TK* mRNAs in some tissues (20). Intron deletion mutations of the chicken *TK* gene were made by combining cDNA and genomic fragments at shared restriction sites (Fig. 1). The mutations were named for the introns that were deleted from the gene. For example, the mutation Δi1-2 lacks introns 1 and 2. Except for the removal of introns, all mutants were otherwise native and used the normal *TK* promoter and polyadenylation signals.

As an initial test of the effect of intron deletion on gene expression, the mutants shown in Fig. 1 were used to transform TK$^-$ L cells to a HAT-resistant phenotype. The transformation efficiency of the different mutations relative to that of the full-length gene was determined in each of several independent transformation series by using the $CaPO_4$ method (8). The results gave no indication of a detrimental effect of intron deletion on gene function (data not shown). However, transformation assays could have obscured a significant effect of intron deletion on *TK* mRNA levels. For example, even if an intronless *TK* gene was 10-fold less effective in generating stable *TK* mRNA, enough mRNA may still be produced to allow growth in HAT medium.

As a more quantitative measure of mutant gene function, *TK* mRNA levels were measured in L cells cotransformed with intron deletion mutations and pKNeo, a plasmid conferring resistance to the drug G418. Resistance to 400 μg of G418 per ml was used to select transformants because HAT might select cells transformed with greater numbers of weaker genes or smaller numbers of stronger genes, thereby obscuring any differences in observed *TK* mRNA levels. Furthermore, by transforming with a 20:1 ratio of *TK*-containing plasmid to pKNeo, cotransformants containing multiple copies of the *TK* gene were selected. Multicopy cotransformants facilitated direct determination of *TK* mRNA levels. Detection of *TK* mRNA was difficult in single-copy HAT-selected transformants or transiently expressing transfectants. All G418-resistant colonies arising from a single transformation were pooled to minimize variability in *TK* expression from individual cotransformants.

TK mRNA levels in polyclonal cotransformant populations were determined by a quantitative RNase protection

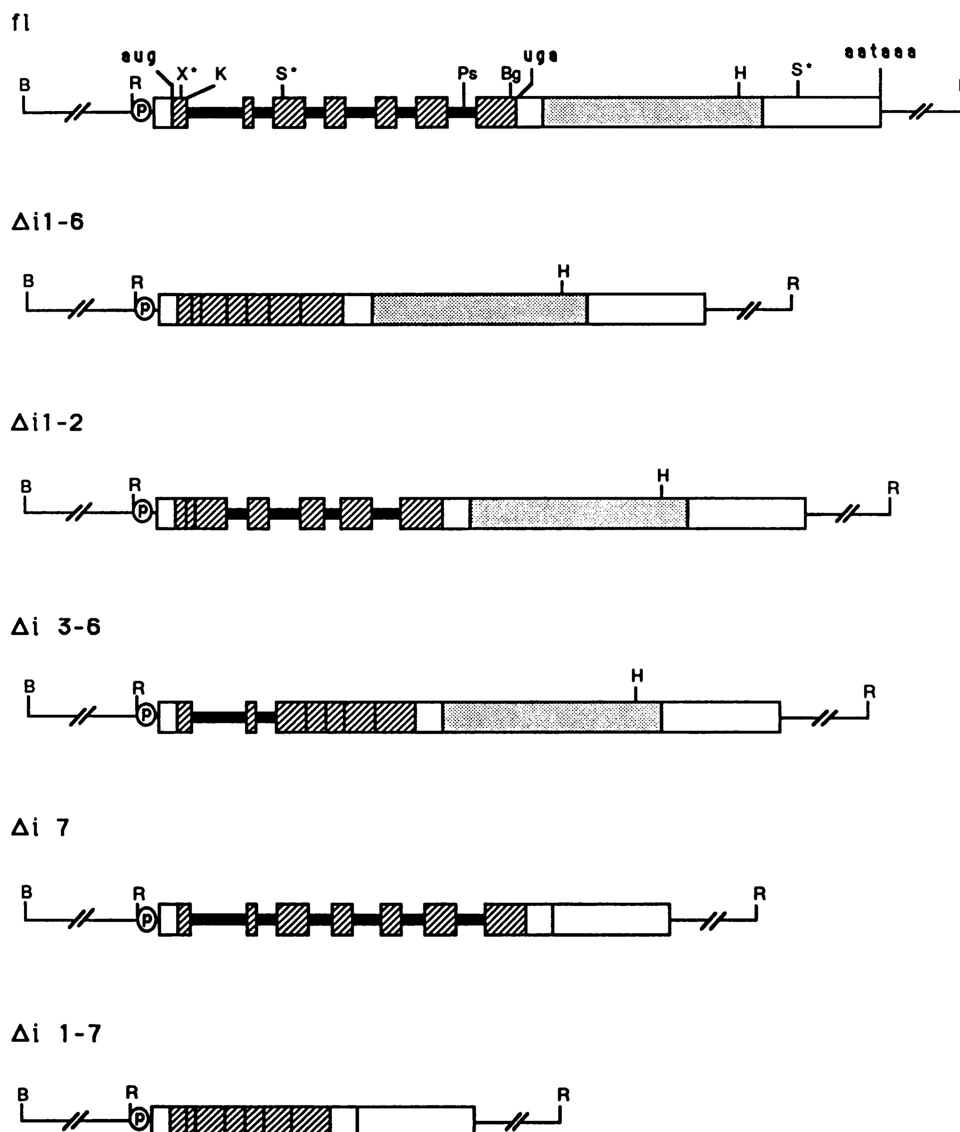

FIG. 1. Intron deletion mutations of the chicken *TK* gene. Hatched regions designate the protein-coding domain, open bars are exons, solid bars are introns, and the stippled region is an intron removed from the 3′ nontranslated region during the biogenesis of rare messages in certain tissues. Letters represent the following restriction enzyme recognition sites: B, *Bam*HI; R, *Eco*RI; X, *Xho*I; K, *Kpn*I; S, *Sst*I; P, *Pst*I; Bg, *Bgl*II; H, *Hin*dIII. The hexanucleotide AAUAAA designates the polyadenylation signal. The figure is drawn to scale; in the full-length *TK* gene (fl), the distance between *Bgl*II and *Hin*dIII is 772 base pairs. Asterisks signify nonunique restriction enzyme sites. All genes extend from a synthetic *Bam*HI linker 775 base pairs upstream from the start codon to a synthetic *Eco*RI site 2,130 base pairs downstream from the stop codon.

assay (9) using total RNA isolated by the method of Glisin et al. (7). Figure 2 diagrams the RNase protection strategy and shows a representative quantitation gel. A sample of the undigested 214-base synthetic RNA probe was loaded in lane 8. Hybridization of this probe to *TK* mRNA and subsequent digestion with RNase resulted in protection of a 147-base exonic fragment. Lanes 9 to 15 contain RNAs isolated from cells cotransformed with full-length *TK*, Δi1-6, Δi3-6, Δi1-2, Δi1-7, Δi7, and no *TK* (pKNeo only), respectively. To allow calculation of absolute *TK* mRNA levels in experimental samples, lanes 1 to 6 were loaded with known amounts of synthetic *TK* mRNA, generated by using an SP6-based in vitro transcription system. In the transformation series shown in Fig. 2 (transformation series 9), Δi1-6 and Δi1-7 transformants (lanes 10 and 13) had less than half as much *TK* mRNA as full-length *TK* transformants (lane 9). This result, seemingly suggestive of an intron requirement, was misleading. When *TK* mRNA molecules per cell were measured in several additional transformation series (Table 1), no consistent detrimental effect of intron deletion was detected.

The number of *TK* mRNA molecules per cell varied considerably between different transformation series (Table 1). Similar variability was observed when *TK* enzyme activity levels were measured (data not shown). Variability in *TK* mRNA and enzyme levels could be due to random differences in gene copy number. This variability could obscure an effect of intron deletion on gene function. Furthermore,

TABLE 1. Chicken *TK* mRNA and gene copy number in mouse L cells transformed with intron delection mutations[a]

Transformation series	No. of *TK* mRNAs/cell						No. of *TK*	
	FL[b]	Δi1-6	Δi1-2	Δi3-6	Δi7	Δi1-7	FL[b]	Δi1-6
9	168	80	136	158	216	59	22	38
10	35	80	—	137	124	134	—	10
11	128	184	193	231	343	—	42	30
12a	40	152	152	103	20	205	6	20
12b	43	127	122	66	11	103	—	83
Mean ± SD	83 ± 61	125 ± 45	151 ± 31	139 ± 62	143 ± 140	125 ± 61	23 ± 19	36 ± 28

nonrandom, preferential integration or stability of intron deletion mutations could compensate for and mask negative effects of intron deletion on gene function.

To determine whether intron deletion mutations were rearranged or preferentially integrated during transformation, *TK* gene copy number and integrity in cotransformants were analyzed by Southern blotting (24). A representative blot of transformant DNA is shown in Fig. 3. In all of the samples, bands of the size expected for the input gene were evident. Thus, intron deletion neither enhanced nor inhibited rearrangement of the gene during the transformation process. To obtain gene copy number, the intensities of the sample bands in Fig. 3 (lanes 5 to 10) were compared with a standard curve generated with chicken liver DNA (lanes 1 to 3). Gene copy number data for five transformation series are summarized in Table 1. No consistent difference in integration efficiency was observed, although considerable random variation in gene copy number was evident. The random differences in gene copy number contributed to the variability in *TK* mRNA levels per cell. When *TK* mRNA levels were normalized to gene copy number levels (Table 1), much of the variability between transformation series was eliminated. However, even with normalization on a per gene basis, no apparent effect of intron deletion on *TK* mRNA expression was observed.

A final approach used to assess the effect of intron removal on gene expression was to include an internal control gene in each transformation. Individual transformants are thought to integrate exogenous DNA as a single concatemeric structure (22). An internal control would allow mRNA expression to be normalized for differentially active

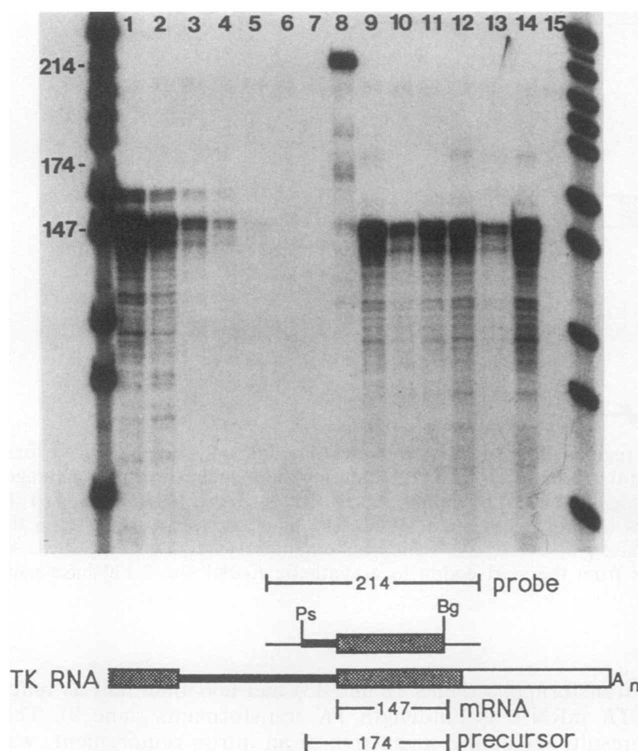

FIG. 2. Representative quantitation gel used to determine absolute levels of *TK* mRNA in mouse L-cell cotransformants. Lanes: 1 to 6, SP6-generated synthetic *TK* mRNA standards, starting at 1.1 fmol and declining in threefold intervals; 7, control digestion with 10 μg of *S. cerevisiae* RNA; 8, nondigested probe; 9 to 15, digestions with 10 μg of RNA from full-length *TK*, Δi1-6, Δi3-6, Δi1-2, Δi1-7, Δi7, and pKNeo-only transformants, respectively. Markers are *Msp*I-digested pBR322. The diagram below illustrates the locations and sizes of the probe and the expected protected fragments. Boxes indicate mRNA sequences, thick lines are intronic sequences, and thin lines are plasmid sequences. The protein-coding region is shaded.

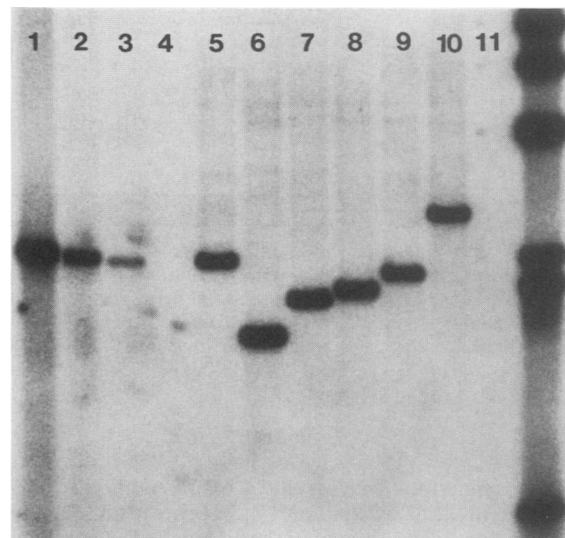

FIG. 3. Representative Southern blot used to determine *TK* gene structure and copy number in mouse L-cell cotransformants. Lanes: 1 to 3, 10, 3, and 1 μg of chicken liver DNA, respectively; 5 to 11, 1 μg of *Hin*dIII- and *Eco*RI-digested DNA from full-length *TK*, Δi1-6, Δi1-2, Δi3-6, Δi7, Δi1-7, and pKNeo-only transformants, respectively. Markers are *Hin*dIII-digested lambda DNA. The blot was probed with a nick-translated *Kpn*I-*Bgl*II fragment of the chicken *TK* gene. Band intensities were compared by laser densitometry. Gene copy number per cell was calculated in light of the DNA contents of mouse and chicken cells (10 and 2.6 pg, respectively) and on the assumption that there were two *TK* genes per chicken cell.

TABLE 1—*Continued*

genes/cell				No. of *TK* mRNAs/gene					
Δi1-2	Δi3-6	Δi7	Δi1-7	FL[b]	Δi1-6	Δi1-2	Δi3-6	Δi7	Δi1-7
26	25	22	19	7.6	2.1	5.2	6.3	9.8	3.1
125	9	8	40	—	8.0	—	15.2	15.5	3.4
27	56	31	—	3.0	6.1	7.1	4.1	11.0	—
13	8	5	21	6.7	7.6	11.7	12.9	4.0	9.8
60	16	4	42	—	1.5	2.0	4.1	2.8	2.5
50 ± 45	23 ± 20	14 ± 12	31 ± 12	5.8 ± 2.4	5.1 ± 3.1	6.5 ± 4.0	8.5 ± 5.2	8.6 ± 5.2	4.7 ± 3.4

[a] *TK* mRNA and gene copy levels were determined as described in the legends to Fig. 2 and 3, respectively. —, No data available.
[b] FL, Full-length *TK* gene.

chromosomal integration sites, as well as for gene dosage. The internal control gene (*2050Tx*) contains introns 1 to 6 but is truncated within exon 7 and joined to the herpes simplex virus *tk* polyadenylation signals (Fig. 4, bottom). It produces an mRNA that protects only 46 bases of the probe used in the RNase protection assay. Southern blot analyses confirmed that the internal control gene and test gene were present in the transformants in the same 1:1 ratio as in the $CaPO_4$ transfection mixture (data not shown). A representative RNase protection gel of two transformation series using the internal control gene is shown in Fig. 4. The usefulness of the internal control gene was apparent for transformation series 12. If the *TK* mRNA produced from Δi7 (at 147 bases) was examined alone, one might conclude that deleting intron 7 was detrimental to *TK* gene expression (compare Δi7 with the full-length *TK* gene). However, the level of mRNA produced from the internal control (at 46 bases) was also very low in Δi7. When normalized with the internal control, the efficiency of mRNA production was about the same for Δi7 and the full-length *TK* gene. Table 2 shows the relative efficiency of mRNA production in four transformation series when the internal control gene was used to normalize expression. The efficiency of mRNA production by the intron deletion constructs varied less than twofold from that of the full-length gene. No detrimental effect of intron deletion was evident.

On the basis of quantitative *TK* mRNA measurements, normalized per cell, per gene, or per internal control, introns were inconsequential to the expression of chicken *TK* mRNA in mouse L cells. Our results indicate that the biogenesis of stable *TK* mRNA is not dependent on passage through an RNA splicing pathway, as has been suggested for simian virus 40 16S mRNA (11–13, 15). Our results also suggest that *TK* gene expression is not dependent on transcriptional regulatory elements located within introns, as has been demonstrated for certain eucaryotic genes (1, 6, 23). In this regard, it should be mentioned that a set of three SP1 binding site consensuses are located within introns 1 and 2 of the chicken *TK* gene (18). These sites are missing in intron deletion mutations Δi1-6, Δi1-2, and Δi1-7, and yet *TK* mRNA is generated efficiently in mouse L cells transformed with these templates.

Our results conflict with earlier transformation analyses, which suggested an intron requirement for mouse *DHFR* and hamster *TK* expression (5, 16, 17). Although chicken *TK* may differ from rodent *TK* and *DHFR* with respect to a role for introns in efficient mRNA formation (perhaps because of the great difference in intron size), we suspect that parameters other than mRNA-generating capacity may have affected the transformation efficiency in these earlier studies.

The expression of three widely divergent eucaryotic genes has been shown to be independent of RNA splicing or

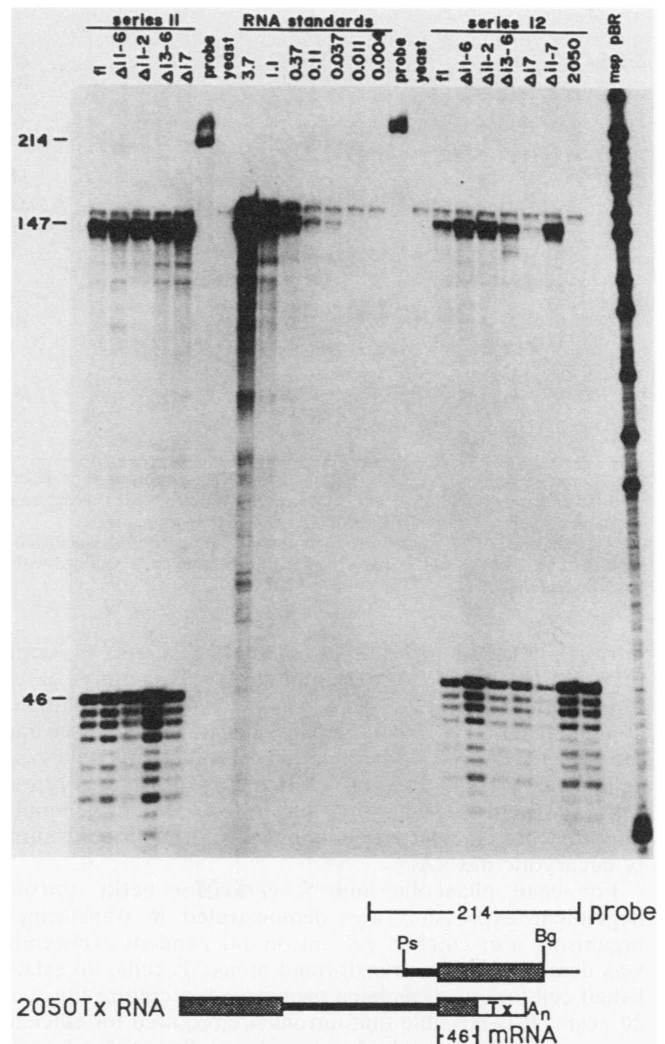

FIG. 4. Efficiency of *TK* mRNA production relative to a positive control gene in mouse L-cell cotransformants. With pKNeo as the selected gene, TK^- L cells were cotransformed with equimolar amounts of each intron deletion mutation and an internal control gene, *2050Tx*. The diagram below illustrates the RNase protection strategy for the internal control gene. Boxes indicate mRNA sequences, thick lines are intronic sequences, and thin lines are plasmid sequences. The protein-coding region is shaded, and Tx indicates the herpesvirus *tk* polyadenylation signals. fl, Full-length *TK*; yeast, *S. cerevisiae*.

TABLE 2. Efficiency of chicken *TK* mRNA production by intron deletion mutations relative to that of an internal control gene

Gene construct	Transformation series	Expression of mutant gene of *TK* mRNA/cell)[a]	Expression (fold) of control gene[b]	Efficiency (fold) of mRNA production by mutant gene (mean ± SD)[c]
Full-length *TK*	11	126.6	1.00	1.00
	12a	40.6	1.00	1.00
	12b	43.7	1.00	1.00
	13	6.8	1.00	1.00
Δi1-6	11	182.3	1.37	1.05
	12a	152.1	9.67	0.39
	12b	126.5	2.13	1.36
	13	12.7	3.12	0.60 (0.85 ± 0.44)
Δi1-2	11	194.2	1.08	1.42
	12a	152.9	2.19	1.72
	12b	121.9	1.00	2.79
	13	72.3	11.05	0.97 (1.73 ± 0.77)
Δi3-6	11	231.9	1.98	0.93
	12a	105.2	4.45	0.58
	12b	66.6	0.95	1.60
	13	23.4	6.33	0.55 (0.92 ± 0.49)
Δi7	11	343.4	0.84	3.23
	12a	19.2	0.52	0.91
	12b	11.9	0.32	0.85
	13	55.6	5.01	1.64 (1.66 ± 1.10)
Δi1-7	12a	207.3	10.48	0.49
	12b	105.3	2.37	1.02
	13	6.0	2.00	0.44 (0.65 ± 0.32)
pKNeo	12a	ND[d]	0.19	ND
	12b	ND	2.06	ND
	13	ND	1.70	ND

[a] *TK* mRNA level per vell was determined by RNase protection as described in the legend to Fig. 4.
[b] Efficiency of expression of the *2050Tx* internal control gene was determined by the intensity of the 46-base RNA signal (see the legend to Fig. 4); values are normalized to the 46-base signal in full-length *TK* transformants.
[c] The efficiency of *TK* mRNA production by mutant genes was calculated by dividing the number of *TK* mRNAs per cell by the efficiency of expression of the positive control gene; values are normalized to the efficiency obtained with full-length *TK* in each transformation series.
[d] ND, Not detectable.

intronic information: bean phaseolin (4), *S. cerevisiae* actin (21), and chicken *TK* (the present study). These three cases represent the only studies in which intron deletion mutations containing native 5′ and 3′ flanking sequences were used and the efficiency of mRNA production by mutant and wild-type cellular genes was directly determined. The studies represent a consensus, suggesting that introns are not generally important for efficient production, transport, or stabilization of eucaryotic mRNA.

For bean phaseolin and *S. cerevisiae* actin, intron-dependent expression was demonstrated in transformed organisms. For chicken *TK*, intron-independent expression was demonstrated in transformed mouse L cells, an established cell line that has been propagated in culture for over 20 years. It is possible that introns are required for efficient gene expression in euploid mammalian cells but that L cells have overcome this requirement during the process of immortalization or during prolonged adaptation to in vitro conditions. To answer this question, the functionality of intron deletion mutations must be analyzed in transgenic organisms, in finite cell lines, or in established cell lines that display properties more characteristic of cells in vivo.

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